

Concentration dependent antioxidant/pro-oxidant activity of curcumin Studies from AAPH induced hemolysis of RBCs

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ABSTRACT

The antioxidant properties of curcumin have been studied by evaluating its ability to protect RBCs from AAPH (2,2'-azobis (2-amidinopropane) hydrochloride) induced oxidative damage. RBCs are susceptible to oxidative damage, resulting in peroxidation of the membrane lipids, release of hemoglobin (hemolysis), release of intracellular K⁺ ions and depletion of glutathione (GSH). In this paper, lipid peroxidation, hemolysis and K⁺ ion loss in RBCs were assessed respectively by formation of thiobarbituric acid reactive substances (TBARS), absorbance of hemoglobin at 532 nm and flame photometry. The treatment of RBCs with curcumin showed concentration dependant decrease in level of TBARS and hemolysis. The IC₅₀ values for inhibition of lipid peroxidation and hemolysis were estimated to be 23.2 ± 2.5 and 43 ± 5 μM respectively. However in contrast to the above mentioned effects, curcumin in similar concentration range, did not prevent release of intracellular K⁺ ions during the process of hemolysis, rather curcumin induced its release even in the absence of hemolysis. The ability of curcumin to prevent oxidation of intracellular GSH due to hemolysis showed mixed results. At low concentrations of curcumin (<10 μM) it prevented GSH depletion and at higher concentrations, the GSH levels decreased gradually. Curcumin scavenges the peroxy radical generated from AAPH. Based on these results, it is concluded that curcumin exhibits both antioxidant/pro-oxidant activity, in a concentration dependent manner.

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1. Introduction

Aerobic organisms generate reactive oxygen species (ROS) such as hydroxyl radicals, superoxide, peroxy radicals etc. during normal metabolism [1,2]. These ROS when produced in excess can cause harmful effects on living cells, resulting into irreparable damage to cellular macromolecules such as lipids, proteins, nucleic acids etc. [1,2]. Curcumin (diferuloylmethane), a dietary pigment responsible for the yellow color of turmeric, has been used in traditional medicine [3–5]. Extensive research within the past one decade has confirmed that

curcumin possesses antioxidant activity and mediates anti-inflammatory effects [6–10]. Curcumin has been shown to inhibit lipid peroxidation, and effectively scavenge superoxide and peroxy radicals [7]. It also shows anti-tumor activity, by suppressing the proliferation of a wide variety of tumor cells [3–5,11,12]. It has been known to upregulate several important antioxidant genes such as HO-1, γ-GCS within cells during oxidative stress [6,9,13,14]. In contrast to these studies some reports suggest that curcumin shows DNA damaging property and leads to induction of apoptosis in cells [15,16]. Curcumin-mediated apoptosis was closely related to the increase in intracellular ROS [8,17–19]. All these reports suggest that curcumin exhibits both antioxidant and pro-oxidant activities in different cells. Therefore in this present work, we have investigated the differential antioxidant/pro-oxidant behavior of curcumin by following

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its ability to protect human RBCs from free radical induced damages. RBCs are enucleated cells, containing polyunsaturated fatty acids in their cell membrane [20]. The major protein component found within these RBCs is the red pigment hemoglobin (Hb) [20]. RBCs are fragile cells, and are highly susceptible to free radical induced damage of cell membrane (lipid peroxidation) leading to leakage of Hb (hemolysis) from within [20–23]. K^+ ions present within the cell, which maintains the osmotic balance, is prone to leakage following hemolysis [23,24]. Therefore, oxidative hemolysis of RBCs and its protection by compounds act as a good model system to screen the test compounds for their antioxidant/pro-oxidant behavior [20,23,25–27]. This model is simple to study and obtain easily accessible results [20]. Hemolysis, was initiated by the peroxy radicals generated by thermal decomposition of an azo compound 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) in the presence of oxygen [20,28]. Since ROS can influence the reduced glutathione (GSH) concentration in cells, GSH levels were also monitored following hemolysis [22,23]. There are a few reports in literature on effect of curcumin on AAPH induced hemolysis of erythrocytes and LDL oxidation [29,30]. However in the present study, hemolysis is monitored by different markers like release of hemoglobin, lipid peroxidation, K^+ ion loss, change in GSH levels and finally the results correlated with the scavenging kinetics of AAPH peroxy radicals by curcumin.

2. Materials and methods

Curcumin, dimethyl sulphoxide (DMSO), thiobarbituric acid (TBA), butylated hydroxy toluene (BHT), Tris base, 2,2'-azobis(2-amidinopropane) hydrochloride, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), ethylene diamine tetra acetic acid (EDTA) were of the best purity available and obtained from commercial sources. All the other chemicals were of analytical grade. Wherever required solutions were prepared in nano-pure water and autoclaved. Absorption spectra were recorded on Hitachi spectrophotometer (model U 2001).

2.1. Preparation of RBCs

Blood samples were obtained by venipuncture from healthy volunteers with strict adherence to the ethical guidelines laid down by the institutional animal ethics committee of Bhabha Atomic Research Centre. Blood was collected in heparinised tubes and centrifuged for 10 min at $1000 \times g$ and $4^\circ C$ using a cold centrifuge (Remi compufuge, CPR-24). Samples were washed three times with a phosphate buffered saline (PBS: NaCl 150 mM, KH_2PO_4 0.58 mM and Na_2HPO_4 3.4 mM, pH 7.4). Plasma and buffy coat were carefully removed by aspiration after each washing. RBCs were finally suspended in the buffer solution to obtain a hematocrit of approximately 50%, stored at $4^\circ C$ and were used within 6 h. All the experiments were carried out in triplicate and the results are presented as means \pm S.E.M., $n=3$.

2.2. Measurement of hemolysis

Hemolysis of RBCs was carried out by mixing 5% suspension of RBCs in PBS with AAPH solution (final concentration 50 mM). This reaction mixture was incubated for 3 h at $37^\circ C$ with gentle shaking. The extent of hemolysis was determined spectrophotometrically by measuring the absorbance of hemolysate at 540 nm as described previously [21]. For reference, RBCs were treated with distilled water and the absorbance of the hemolysate at 540 nm was used as 100% hemolysis. To test the effect of curcumin on hemolysis, RBCs were pre-incubated with varying concentration of curcumin at $37^\circ C$ for 30 min, washed twice with cold PBS and then subjected to hemolysis. This 30 min incubation time would ensure that curcumin is taken up by the RBCs.

2.3. Membrane lipid peroxidation

Lipid peroxidation was assessed by measuring the thiobarbituric acid (TBA) reactive substances (TBARS). For this 5% suspension of RBCs in PBS (pH 7.4) was incubated under air atmosphere with varying concentration of curcumin at $37^\circ C$ for 30 min, washed twice with cold PBS and then incubated into a PBS solution of AAPH (50 mM) to initiate membrane damage. After incubation at room temperature for 3 h, the system was centrifuged at $1500 \times g$ for 10 min and the pellet was resuspended in to $300 \mu l$ PBS (pH 7.4) to which $900 \mu l$ of TBA reagent (0.375% thiobarbituric acid, 0.25 M HCl, 15% trichloroacetic acid and 6 mM EDTA) was added and after further treatment, TBARS were estimated by measuring the absorbance at 535 nm [23] and expressed as per mg of hemoglobin.

The IC_{50} value i.e. the concentration of curcumin required to inhibit, hemolysis or lipid peroxidation by 50%, was determined by plotting the percent hemolysis, or TBARS levels respectively as a function of curcumin concentration, and from the plot, the concentration of curcumin required to reduce the activity by 50% was identified.

2.4. Measurement of K^+ ion loss

Pecked RBCs were suspended in 3 ml of 10 mM PBS, containing varying amount of curcumin to give 0.5% hematocrit. After 30 min of incubation at $37^\circ C$ and two washes with cold PBS, AAPH (50 mM) was added and this system was incubated at room temperature for 3 h. After centrifugation at $1500 \times g$ for 10 min, the concentration of K^+ ion was measured in the supernatant using flame photometry (Chemito AA 203, atomic absorption spectrophotometer with programmable flame control unit) [23]. For reference of 100% intracellular K^+ ion, a sample of RBCs was hemolysed in distilled water and the K^+ ion concentration was determined in the supernatant after centrifugation.

2.5. Glutathione estimation

The concentration of reduced glutathione in RBCs was determined using 5,5'-dithiobis-2-nitrobenzoic acid according to the reported method [22,23,31]. In a typical experiment, 5% suspension of RBCs in PBS (pH 7.4) was

subjected to hemolysis by treatment with AAPH for 3 h, in presence of different concentrations of curcumin. The hemolysate was precipitated using 10% TCA, centrifuged and the supernatant used for the estimation of free non protein bound reduced GSH and expressed as nmol DTNB per mg of hemoglobin.

2.6. Study of the reaction of curcumin with AAPH

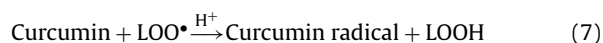
The rate constant for the reaction of curcumin with AAPH was estimated by following the time dependent changes in the absorbance of curcumin at 435 nm in presence of AAPH and fitting the absorption–time plot to first order exponential function as given below:

$$A_t = A_0 e^{-(k_{\text{obs}}t)} \quad (1)$$

here A_0 and A_t represent the respective initial absorbance and absorbance at given time “ t ” at 435 nm. k_{obs} is the observed first order decay rate constant. This k_{obs} was estimated at four different curcumin concentrations (10–50 μM) and the average value of k_{obs} divided by curcumin concentration is considered as the bimolecular rate constant for the reaction of AAPH with curcumin.

3. Results

When AAPH is added as initiator, it decomposes at physiological temperature (37 °C) in aqueous solutions to generate alkyl radical (R^{\bullet}), which in presence of oxygen is converted to the corresponding peroxy radicals ($R^{\bullet}OO^{\bullet}$) Eqs. (2) and (3). At 37 °C in neutral water, the half-life of AAPH is about 175 h and generates radicals at a rate of 1.3×10^{-6} [AAPH]/s [28]. These peroxy radicals induce oxidation of polyunsaturated lipids (LH) in RBC membranes causing a chain reaction known as lipid peroxidation Eqs. (4–6). As a result of this lipid peroxidation, the RBC membrane undergoes quick damage and losses its integrity, leading to the release of hemoglobin (hemolysis) and intracellular K^+ ions. When compounds like curcumin are present and if they can scavenge peroxy radicals, and convert them to non-reactive species Eq. (7), the hemolysis can be inhibited.



where R^{\bullet} is $-C(\text{Me})_2-C(\text{NH}_2)=\text{NH}_2^{\bullet}$

3.1. Inhibition of AAPH induced hemolysis in human RBCs by curcumin

In the absence of AAPH, RBCs were stable and the hemolysis was negligible. When aqueous suspension of RBCs was incubated with AAPH, about 53% of hemolysis was observed. Fig. 1(a) shows variation in percent hemolysis in

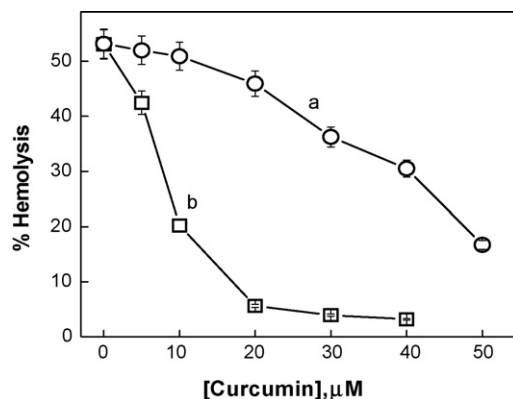


Fig. 1. (a) Variation in percent hemolysis in human RBCs pre-incubated with different concentrations of curcumin and then subjected to hemolysis by 50 mM AAPH for 3 h. (b) Variation in percent hemolysis in human RBCs incubated simultaneously with 50 mM AAPH and different concentrations of curcumin for 3 h. Results are presented as means \pm S.E.M., $n = 3$.

RBCs pre-incubated with increasing concentrations of curcumin (5–50 μM) for 30 min and subjected to hemolysis. It is evident from Fig. 1(a) that the percent hemolysis gradually decreased with increasing concentration of curcumin, from which the IC_{50} value, was found to be $43 \pm 5 \mu\text{M}$. Fig. 1(b) shows variation in percent hemolysis in RBCs incubated simultaneously with increasing concentrations of curcumin (5–50 μM) and AAPH. It is evident from Fig. 1(b) that the percent hemolysis progressively decreased with increasing concentration of curcumin, from which the IC_{50} value was found to be $8 \pm 0.6 \mu\text{M}$. The percent hemolysis in RBCs incubated with curcumin (5–50 μM) in the absence of AAPH was almost identical to that of control sample indicating curcumin itself could not induce hemolysis.

3.2. Inhibition of AAPH induced lipid peroxidation in human RBCs by curcumin

Fig. 2 shows variation in TBARS in RBCs after subjecting to AAPH induced damage in the presence and absence of different concentrations of curcumin. The level of TBARS

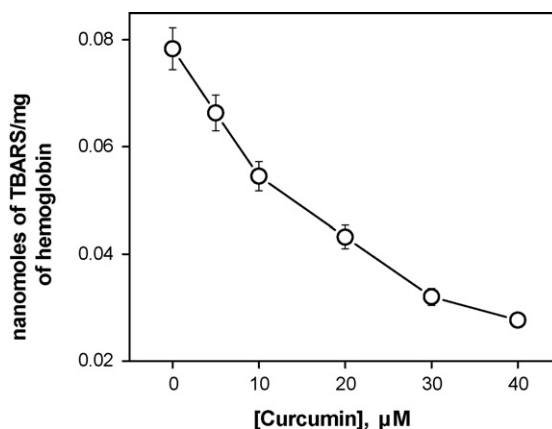


Fig. 2. Variation in TBARS estimated for lipid peroxidation in human RBCs induced by 50 mM AAPH after incubation for 3 h at different concentrations of curcumin. Results are presented as means \pm S.E.M., $n = 3$.

was significantly increased after incubation of RBCs with AAPH as compared to the control sample. In the presence of curcumin, there was gradual decrease in TBARS formation and this inhibition increased with increasing curcumin concentration from 5 to 40 μM , from which the IC_{50} value was found to be $23.2 \pm 2.5 \mu\text{M}$. The incubation of RBCs with curcumin in the absence of AAPH did not show any significant change in the level of TBARS as compared to control sample.

3.3. Inhibition of AAPH induced K^+ ion leakage in human RBCs by curcumin

In the absence of AAPH, the RBCs were stable and the K^+ ion leakage was negligible. When aqueous suspension of RBCs was incubated with AAPH, about 79% of K^+ ion leakage from RBCs pre-incubated with increasing concentrations of curcumin (5–40 μM) for 30 min and subjected to hemolysis. It is evident from Fig. 3 that the percent K^+ ion leakage is lower in curcumin pretreated samples compared to the control sample. However in each case, the K^+ ion leakage increased with increasing concentration of curcumin. Inset of Fig. 3 shows variation in percent K^+ ion loss in RBCs after incubation with different concentrations of curcumin (5–100 μM) for 3 h in the absence of AAPH. From the figure it is clear that the percent K^+ ion loss is significantly higher in curcumin treated samples as compared to control sample. The percent K^+ ion loss was almost identical (nearly 47%) at all the concentrations of curcumin (5–100 μM) tested in present study.

3.4. Effect of curcumin on GSH levels in RBCs after hemolysis

Fig. 4 shows change in GSH level in RBCs after treatment with AAPH and also in presence of increasing concentration of curcumin (5–40 μM). The normal basal level of GSH in RBCs was found to be $2.74 \pm 0.05 \text{ nmol/mg}$ of hemoglobin

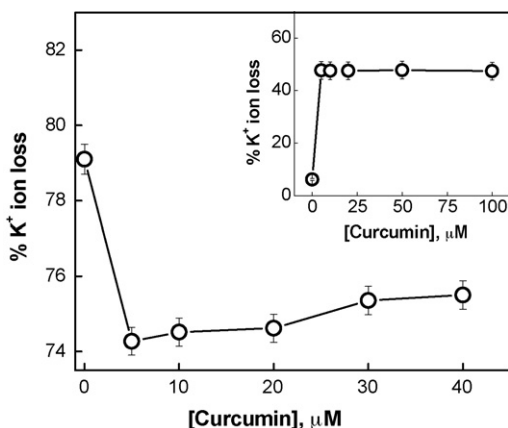


Fig. 3. Variation in K^+ ion loss estimated by flame photometry in human RBCs incubated with 50 mM AAPH for 3 h in presence of different concentrations of curcumin. Inset shows variation in K^+ ion loss in human RBCs incubated with different concentrations of curcumin without any added AAPH. Results are presented as means \pm S.E.M., $n = 3$.

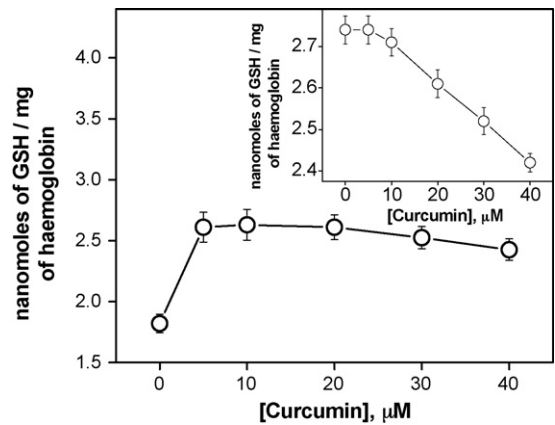


Fig. 4. Variation in glutathione (GSH) levels in human RBCs incubated with 50 mM AAPH for 3 h in presence of different concentrations of curcumin. Inset shows variation in glutathione levels in human RBCs incubated with different concentrations of curcumin without any added AAPH. Results are presented as means \pm S.E.M., $n = 3$.

and after incubation with AAPH, the GSH level reduced to about $1.82 \pm 0.03 \text{ nmol/mg}$ of hemoglobin. Addition of curcumin to this reaction system prevented the reduction in GSH content in a concentration dependant manner up to 10 μM . However at higher concentrations of curcumin treatment, GSH content reduced in a concentration dependant manner. Inset of Fig. 3 shows variation in GSH level in RBCs after incubation with different concentrations of curcumin (5–40 μM) for 3 h in the absence of AAPH. From the figure it is clear that the level of GSH in curcumin treated RBCs was almost constant up to treatment concentration of 10 μM , however beyond that a concentration dependant decrease in level of GSH was observed.

3.5. Reaction of curcumin with AAPH peroxy radicals

Curcumin exhibits a broad absorption spectrum in PBS solution containing 1% DMSO with maximum absorption at $\sim 435 \text{ nm}$, while that of 50 mM AAPH at pH 7 showed absorption maximum at 350 nm, with no absorption at wavelength $> 400 \text{ nm}$ (Fig. 5a and b). On incubating with AAPH, for 30 min the absorption spectrum of curcumin changed significantly and the absorbance due to curcumin decreased completely. Fig. 5c and d, show spectra recorded at two different times 5 and 30 min after incubation with AAPH. Curcumin itself undergoes degradation in aqueous medium and the absorbance decays with the half-life of 96.7 min (Fig. 5e). The decay of curcumin becomes faster in the presence of AAPH with the half-life of 4.5 min. The absorption–time plot (Fig. 5f) shows the decay of the parent curcumin (30 μM) absorption at 435 nm after mixing with AAPH. As seen in the Fig. 5 this is much faster than the self decay of curcumin, therefore the self decay of curcumin can be ignored under these conditions. The rate constant for the reaction between curcumin and AAPH was calculated by following the decay of curcumin at different concentration (10–50 μM) in presence of AAPH. The k_{obs} was obtained by fitting the decay trace to a first order exponential function (Eq. (1)), at a given concentration of curcumin and from which the bimolecular rate constant for the reaction (8) was

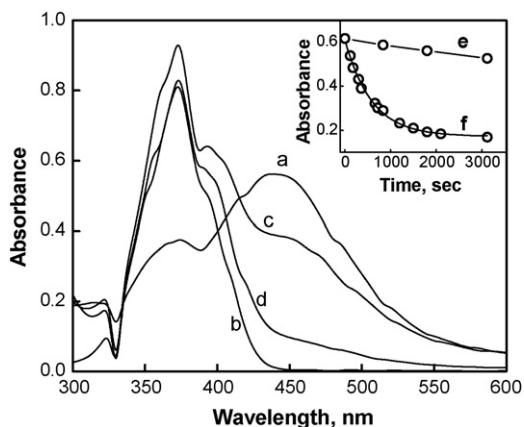


Fig. 5. Absorption spectra of (a) 25 μM curcumin, (b) 50 mM AAPH in PBS buffer containing 1% DMSO. (c) and (d) correspond to the spectra obtained on mixing 25 μM curcumin with 50 mM AAPH after 5 and 30 min respectively. Inset shows the change in absorbance of 30 μM curcumin at 435 nm as a function of time in (e) the absence and (f) in presence of 50 mM AAPH at pH 7.

estimated to be $63.4 \pm 12.7 \text{ M}^{-1} \text{ s}^{-1}$. Due to slow rate limiting decomposition of AAPH this value may not be a true representation of the rate constant for the overall reaction (8) but it certainly confirms direct reaction of AAPH radicals with curcumin.



Earlier using pulse radiolysis technique, it has been reported that curcumin reacts with trichloromethyl peroxy radical and lineolic peroxy radical with rate constant of 5×10^8 and $5.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ respectively [7]. The low apparent rate constant with AAPH is mainly due to slow rate limiting release ($1.3 \times 10^{-6} \text{ s}^{-1}$) of peroxy radicals from AAPH decomposition [28].

4. Discussion

Hemolysis of human RBCs is a very good model for studying free radical induced oxidative damage to membranes and to evaluate the antioxidant activity of new compounds [20,23,25–27]. Therefore to evaluate the antioxidant activity of curcumin, lipid peroxidation of the membrane fatty acids, loss of hemoglobin and release of intracellular K^+ ions have been estimated in human RBCs treated with AAPH [20,28]. The results obtained from these studies indicate that curcumin by itself did not cause either lipid peroxidation or hemolysis to RBCs, and showed significant protection from AAPH induced lipid peroxidation and hemolysis. Curcumin has also been shown to react with the peroxy radicals generated from AAPH. Thus the overall effect of curcumin on AAPH induced protection of RBCs may be either due to direct chemical neutralization of peroxy radicals generated from AAPH or its cellular uptake. To find out more about these possibilities hemolysis experiments were carried out in two different conditions. In the first condition, the RBCs were incubated with curcumin for 30 min prior to AAPH addition and in the second condition the RBCs were treated with curcumin and AAPH simultaneously. The IC_{50} values estimated for these two

systems indicated higher value for the former compared to the latter. The results suggest that when RBCs are pre-incubated with curcumin for 30 min, majority of curcumin may have been taken up by the RBCs, leaving very minute amounts of curcumin in the extra-cellular aqueous medium to be scavenged by the peroxy radicals and therefore elevating the IC_{50} value. However when RBCs were treated with curcumin and AAPH simultaneously more curcumin is available to be scavenged by the peroxy radicals, resulting in low IC_{50} value. Therefore in all the further experiments we followed the incubation of RBCs with curcumin prior to AAPH treatment in order to avoid direct chemical neutralization of peroxy radicals by curcumin generated from AAPH.

Like in lipid peroxidation and hemolysis assays, curcumin did not show progressive inhibition of K^+ ion loss and reduced GSH depletion under AAPH treated condition thus making us unable to estimate their IC_{50} values. Curcumin treated samples showed lower percent of K^+ ion loss as compared to AAPH treated samples. However in each case, the percent K^+ ion loss increased with increasing concentrations of curcumin. Therefore to know whether curcumin itself has any effect on K^+ ion loss, we looked at the loss of K^+ ion in only curcumin treated samples. The results clearly suggest that curcumin at concentration as low as 5 μM drastically increased the loss of K^+ ions (47%) from RBCs. With the increasing curcumin concentration, the percent of K^+ ion loss remained almost the same suggesting the saturation effect of curcumin on K^+ ion loss. The ability of curcumin to induce K^+ ion loss may be because of its effect on Na^+/K^+ ion channels present on RBC membranes. This indicates that curcumin may not be acting as a simple antioxidant but probably has a pro-oxidant effect.

GSH is the most abundant thiols present in mammalian cells [22,23,32]. It performs a number of vital cell functions including maintaining the essential thiol status of proteins by preventing oxidation of sulfhydryl groups or by reducing disulphide bonds induced by oxidative stress, or by scavenging free radicals. During oxidative stress, the cellular pool of GSH is depleted. Exogenously applied antioxidants protect GSH levels in cells by preventing them from being consumed in reaction with free radicals [33]. Our results indicate that the GSH levels come down significantly in RBCs after AAPH incubation, but treatment with curcumin in the concentration range from 5 to 10 μM significantly prevents the decrease in GSH level. However further increase in concentration of curcumin showed decrease in GSH level in a concentration dependant manner. Curcumin treatment alone without AAPH did not show much effect on GSH level in the concentration range up to 10 μM , and further increase up to 40 μM resulted in significant decrease of GSH level, probably due to the direct reaction of curcumin with GSH [34]. Some reports suggest that curcumin reacts with ROS and generates less reactive phenoxy radical Eq. (7) [7,33]. Therefore the observed decrease in GSH level in curcumin (>10 μM) pretreated samples after exposure to AAPH could also be due to the excess accumulation of phenoxy radicals and thereby oxidation of cellular GSH to GSSG [33]. Hence this observation indicates that curcumin at higher concentrations (>10 μM) shows pro-oxidant behavior.

Earlier Deng et al. have also shown the protection of RBCs from oxidative hemolysis by curcumin [26]. In this study the authors looked at the inhibition of hemoglobin leakage by curcumin and thus explained the antioxidant activity of curcumin, which is in agreement with our results. In present study, apart from hemoglobin leakage several other sensitive parameters associated with oxidative hemolysis such as K^+ ion leakage and glutathione depletion have been taken in to care therefore making us enable to identify the dual activity of antioxidant and pro-oxidant associated with curcumin. In this respect, it is worth mentioning that most of the *in vivo* studies dealing with curcumin as an anti-tumor agent discuss about its poor bioavailability as low blood levels are achieved after excessive oral administration (8 g/day) [5]. Our results compliment these studies and suggest that at low blood levels, curcumin shows antioxidant activity on RBCs, which is desired along with its anti-tumor activity. In conclusion curcumin shows both antioxidant and pro-oxidant activity in RBCs hemolysis model and at high curcumin concentration, it is the later one, which predominates over the former.

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